Active Transport of Oxalate by Pseudomonas oxalaticus OX1

Dijkhuizen, L.; Harder, W.; Konings, W.N.

Published in:
Archives of Microbiology

DOI:
10.1007/BF00406378

Publication date:
1977

Arch. Microbiol. 115, 223–227 (1977)

Archives of
Microbiology

Active Transport of Oxalate by *Pseudomonas oxalaticus* OX1

L. DIJKHUIZEN, L. GROEN, W. HARDER, and W. N. KONINGS

Department of Microbiology, University of Groningen, Kerklaan 30, Haren (Gr.), The Netherlands

Abstract. Membrane vesicles isolated from oxalate-grown cells of *Pseudomonas oxalaticus* accumulated oxalate by an inducible transport system in unmodified form against a concentration gradient. This accumulation was dependent on the presence of a suitable electron donor system such as ascorbate-phenazine-methosulphate. In the presence of this energy source, steady state levels of accumulation of oxalate were 10–20-fold higher than in its absence. The oxalate transport system involved showed a high affinity for oxalate ($K_m = 11$ μM) and was highly specific. Oxalate transport was not affected by the presence of other dicarboxylic acids, such as malate, succinate and fumarate and only partly inhibited by acetate. The energy requirement for oxalate transport is discussed and it is concluded that this requirement is most likely equivalent to 1 mole of ATP per mole of oxalate.

Key words: *Pseudomonas oxalaticus* OX1 – Active transport – Oxalate – Protonmotive force – Energetics.

Pseudomonas oxalaticus is able to grow on oxalate as the sole source of carbon and energy (Quayle, 1961; Dijkhuizen et al., 1977). The enzymes involved in the initial metabolism of oxalate are cytoplasmic and oxalate can therefore only be utilized after its translocation across the cell envelope.

Since oxalate is a relatively small and highly oxidized molecule its degradation can yield only 1 mole of NADH per mole of oxalate. Any energy required for the uptake of oxalate into the cell will therefore have a significant effect on the overall energy budget of the cell. In order to estimate the net amount of energy generated in the oxidation of 1 mole of oxalate, it is essential to establish the nature of the transport system for oxalate and to estimate the energy required for the translocation of oxalate across the cell envelope.

Until now, little is known about the mechanism of transport of small mono- and dicarboxylic acids. Garland et al. (1975) concluded from their experiments with *Escherichia coli* that formate enters the cell by simple diffusion. This is also the case with acetate (Padan et al., 1976; Boonstra and Konings, 1977). Nothing is known about the uptake of carbon sources such as oxalate, glyoxylate or glycollate, particularly with respect to the requirement for metabolic energy and the involvement of specific carrier systems. It seemed therefore worthwhile to initiate such transport studies in *Pseudomonas oxalaticus* and in this paper we report a study on the mechanism of oxalate transport as investigated in cytoplasmic membrane vesicles obtained from this organism. Evidence is presented for the occurrence of an active transport system for oxalate in *Pseudomonas oxalaticus*.

Abbreviation. PMS = phenazinemethosulphate

MATERIALS AND METHODS

Growth Conditions and Isolation of Membrane Vesicles. *Pseudomonas oxalaticus* OX1 was maintained as described previously (Dijkhuizen and Harder, 1975). The organism was grown in a batch fermenter with a working volume of 2 l (Harder et al., 1974) on the medium described by Dijkhuizen and Harder (1975). The concentration of the carbon sources dipotassium oxalate, sodium formate or sodium lactate, used was 50 mM. During growth the pH was kept constant at 7.5 by the automatic addition of 2 N solutions of oxalic acid, formic acid, and sulphuric acid, respectively. The organisms were harvested at the end of the logarithmic growth phase. Membrane vesicles were isolated as described by Kaback (1971), and were stored in small aliquots (0.5 ml) in liquid nitrogen.

Transport Studies. All uptake experiments were performed at 25° C as described by Matin and Konings (1973), using a final incubation

mixture of 100 µl. The reaction was started by the addition of an electron donor.

Oxygen Consumption. Oxygen consumption was measured polarographically in 3.0 ml reaction mixture at 25° C as described by Konings (1975). *Protein* was determined by the method of Lowry et al. (1951).

Thin Layer Chromatography. The purity of ^{14}C-oxalate and its R_f-value was determined by thin layer chromatography as described by Matin and Konings (1973). This method was also used for analyzing possible products formed from oxalate after accumulation by the membrane vesicles. For that purpose the radioactive material was extracted from the membrane vesicles, freeze-dried and dissolved in water at a concentration suitable for thin layer chromatography.

Chemicals. The ^{14}C-labeled compounds, [U-^{14}C]oxalate (74 mCi/mMol) and L-[U-^{14}C]aspartate (230 mCi/mMol) were obtained from the Radiochemical Centre, Amersham, England.

RESULTS

Membrane vesicles isolated from oxalate-grown cells of *Pseudomonas oxalaticus* accumulated oxalate (Fig. 1). In the presence of the electron donor system ascorbate-phenazinemethosulphate (Konings and Freese, 1971) a high initial rate of oxalate uptake was observed. Steady state levels of accumulation were reached that were 10–20-fold higher than the levels obtained in the absence of an energy source. A stimulation of oxalate uptake was also observed with NADH, but this potentially physiological electron donor for the electron transport chain was far less effective than the artificial electron donor. Other potential electron donors such as succinate and formate had no positive effect on the uptake of oxalate. Thin layer chromatography of the radioactive material that accumulated inside the membrane vesicles showed only one spot with the same R_f-value as oxalate. Oxalate appeared to be taken up by an inducible transport system. In contrast with preparations from oxalate-grown cells, vesicle preparations from formate- or lactate-grown organisms did not accumulate oxalate. This was not due to any deficiency in these vesicles since vesicles prepared from cells grown on all three substrates, when energized by ascorbate-PMS or NADH, accumulated amino acids such as L-aspartate at comparable rates (data not shown). No uptake of formate was detectable in vesicles isolated from cells grown on either oxalate or formate. These results indicate that uptake of oxalate by the membrane vesicles of oxalate-grown cells is mediated by an active transport system. Oxalate is accumulated inside the vesicles by an inducible transport system in unmodified form against a concentration gradient and the uptake is dependent on energy supplied by the oxidation of electron donors via the respiratory chain.

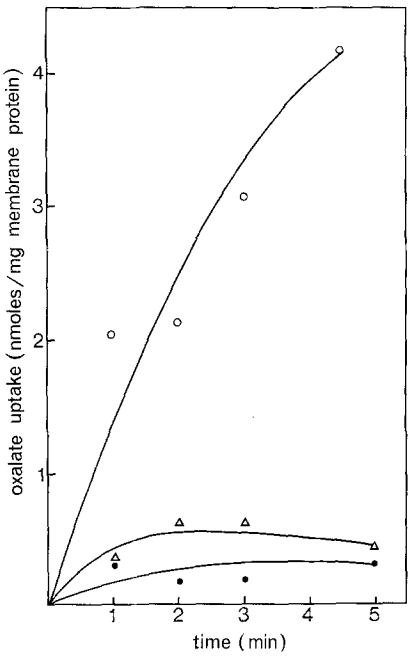

Fig. 1. Transport of oxalate by membrane vesicles from oxalate-grown *Pseudomonas oxalaticus* OX1. ^{14}C-oxalate concentration: 54 µM. Protein concentration: 0.6 mg/ml. ●——● in the absence of energy source; △——△ in the presence of NADH (10 mM); ○——○ in the presence of K-ascorbate (pH 6.6; 10 mM) and PMS (100 µM)

Table 1. Oxidation of various substrates by membrane vesicles isolated from oxalate-grown *Pseudomonas oxalaticus* OX1

Substrate	Concentration (mM)	Oxygen uptake natom × min^{-1} × mg membrane protein
None	–	0
Oxalate	0.33	0
Formate	0.33	50
Succinate	10	80
NADH	5	120

The oxidation rates of various potential electron donors by membrane vesicles of *P. oxalaticus* are given in Table 1. Endogenous oxygen consumption was negligible as was oxygen consumption in the presence of oxalate, indicating that the membrane vesicles were devoid of cytoplasmic components. On the other hand, a high rate of oxygen consumption was observed in the presence of formate, succinate and NADH. Interestingly, the physiological electron donor, NADH, is oxidized at the highest rate by the membrane vesicles, but is a poor energy source for oxalate transport. The observed rapid

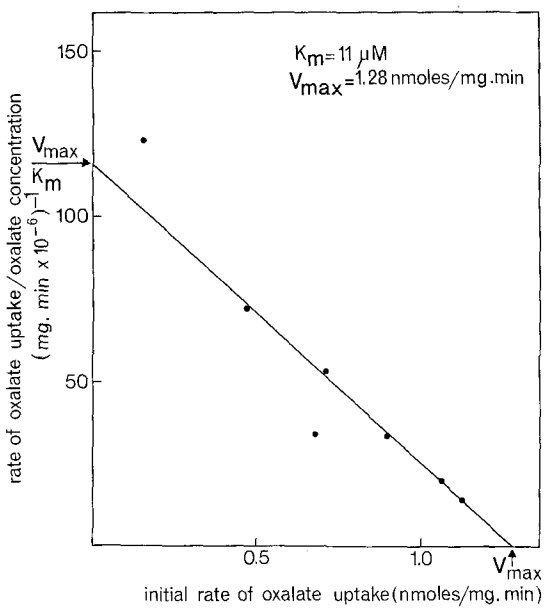

Fig. 2. Hofstee plot of oxalate uptake by membrane vesicles from oxalate-grown *Pseudomonas oxalaticus* OX1

Table 2. Percentage inhibition of the initial rate of oxalate transport by various carboxylic acids in membrane vesicles from oxalate-grown *Pseudomonas oxalaticus* OX1. ^{14}C-oxalate concentration: 54 μM

Inhibitors (1 mM)	Percentage inhibition	Inhibitors (1 mM)	Percentage inhibition
Succinate	10	Glyoxylate	41
Formate	35	Pyruvate	0
Oxalate	98	Glycine	0
Acetate	63	Glycollate	11
Oxamate	62	DL-Malate	20
Malonate	30	Fumarate	8
Citrate	30		

oxidation of formate by the membrane vesicles is probably due to the presence of a membrane-bound formate dehydrogenase (Dijkhuizen et al., unpublished).

The initial rates of oxalate uptake as a function of the oxalate concentration displayed simple Michaelis-Menten kinetics. A Hofstee plot of the data was linear over the concentration range between 2 and 40 μM (Fig. 2). From this plot a K_m value for the oxalate transport system of 11 μM was estimated. This low figure indicates that oxalate is transported by a high affinity carrier system. An increase of the initial rate of oxalate uptake was observed above concentrations of 50 μM. This suggests that in addition to the high affinity system a low affinity system for oxalate transport may be present. Saturation of this system was not obtained even at the highest ^{14}C-oxalate concentration possible in the present experiments (130 μM). This system was not further investigated.

The effect of several structurally and metabolically related carboxylic acids on the initial rate of oxalate transport is summarized in Table 2. It appeared that the high affinity transport system for oxalate was also highly specific. Although the carboxylic acids were added at concentrations 20 times that of oxalate, inhibition of oxalate transport never exceeded 65%. This indicates that the oxalate transport system does not have a significant affinity for the added carboxylic acids when present in concentrations below 1 mM.

DISCUSSION

Evidence is accumulating that electron flow via the respiratory chain results in the generation of a protonmotive force, which, for instance, can be used as the driving force for active transport processes (Mitchell, 1976). The protonmotive force consists of two components: a pH gradient (ΔpH) and a membrane potential ($\Delta\psi$). Cationic compounds probably respond directly to the membrane potential, because of the negative charge on the inside of the membrane, while the driving force in the transport of anionic and neutral compounds is most likely the ΔpH and the total protonmotive force, respectively (Hamilton, 1975; Ramos and Kaback, 1977). Oxalate is a dicarboxylic acid with pK values for the two acidic groups of 1.25 and 4.14, respectively. At the pH values used in the transport experiments (pH 6.6) and at that of the growth medium (pH 7.5) both carboxylic groups are fully dissociated. The carrier for oxalate in unloaded form will be either neutral or negatively charged and the loaded carrier either neutral (driving force ΔpH) or positively charged (driving force ΔpH + $\Delta\psi$). Therefore, under these experimental conditions in order to drive transport of oxalate by the ΔpH, symport with at least two protons is required. Evidence for cotransport with approximately two protons has been presented for the dicarboxylic acids succinate, malate and fumarate in *Escherichia coli* (Gutowski and Rosenberg, 1975). However, the ΔpH appears to be large only when the external pH is comparatively low and decreases to very low values at neutral external pH, whereas $\Delta\psi$ is constant over the pH range 5–9 (Padan et al., 1976; Ramos et al., 1976). This could make it favourable and perhaps inevitable for an organism to use the membrane potential as the main driving force at pH values of 7.5 and higher (Rottenberg, 1976). This can only be attained when the carrier-substrate complex is positively charged which means, in the

case of oxalate, cotransport of at least three protons. In studies with *E. coli* membranes vesicles Ramos and Kaback (1977) experimentally confirmed that transport of anions was driven by the $\Delta\psi$ at pH 7.5.

It is most likely, therefore, that transport of oxalate is accompanied by the symport of at least two protons per molecule. Since the synthesis of ATP requires the translocation from the outside to the inside of probably two (Mitchell, 1966) and at the most four protons (Brand et al., 1976; Papa, 1976; but see also Moyle and Mitchell, 1977), the energy required for the translocation of one molecule of oxalate from outside to inside is equivalent to at least half a molecule of ATP and most likely one molecule of ATP. A similar reasoning can be applied in the case of formate. No active transport system for this monocarboxylic acid was detectable in vesicles of oxalate- or formate-grown *Pseudomonas oxalaticus*. It has been shown in *E. coli* and *Bacillus subtilis* that formate is taken up by simple diffusion of the undissociated molecule (Garland et al., 1975; Boonstra and Konings, 1977). This is most likely also the situation in *P. oxalaticus*. Nevertheless, this diffusion of formate is an energy requiring process because of the cotransport of at least one proton per molecule of formate. Thus, the energy required for the translocation of one molecule of formate across the membrane is most likely equivalent to half a molecule of ATP.

The observation was made that, although membrane vesicles of oxalate-grown *P. oxalaticus* were able to oxidize NADH at a very fast rate (Table 1), NADH was a rather poor energy source for the transport of oxalate (Fig. 1). A comparable situation is found in *E. coli* ML 308-225 membrane vesicles (Stroobant and Kaback, 1975) where NADH is a poor electron donor for active transport because NADH-oxidation does not lead to the generation of an effective protonmotive force (Ramos et al., 1976). A satisfactory explanation has not been offered at this moment.

The results shown in Table 2 indicate that the oxalate transport system is highly specific. The affinity constant of 11 μM for oxalate is of the same order of magnitude as that observed for the C_4-dicarboxylic acid transport system in *B. subtilis* (Bisschop et al., 1975). But, whereas each of the three dicarboxylic acids involved in this system, succinate, L-malate and fumarate, inhibits transport or the other two, oxalate does not inhibit this system. Conversely, in *P. oxalaticus* oxalate transport is not affected by the presence of succinate, malate or fumarate. This indicates that oxalate is transported by a carrier system clearly different from that involved in C_4-dicarboxylic acid transport.

Acknowledgements. The investigations were supported (in part) by the Foundation for Fundamental Biological Research (BION) which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO). We wish to thank Dr. M. Knight for valuable suggestions and correction of the English text.

REFERENCES

Bisschop, A., Doddema, H., Konings, W. N.: Dicarboxylic acid transport in membrane vesicles from *Bacillus subtilis*. J. Bacteriol. **124**, 613–622 (1975)

Boonstra, J., Konings, W. N.: Generation of an electrochemical proton gradient by nitrate respiration in membrane vesicles from anaerobically grown *Escherichia coli*. Eur. J. Biochem. **78**, 361–368 (1977)

Brand, M. D., Reynafarje, B., Lehninger, A. L.: Stoicheiometric relationship between energy-dependent proton ejection and electron transport in mitochondria. Proc. Nat. Acad. Sci. U.S.A. **73**, 437–442 (1976)

Dijkhuizen, L., Harder, W.: Substrate inhibition in *Pseudomonas oxalaticus* OX1: a kinetic study of growth inhibition by oxalate and formate using extended cultures. Antonie van Leeuwenhoek **41**, 135–146 (1975)

Dijkhuizen, L., Wiersma, M., Harder, W.: Energy production and growth of *Pseudomonas oxalaticus* OX1 on oxalate and formate. Arch. Microbiol. **115**, 229–236 (1977)

Garland, P. B., Downie, A. J., Haddock, B. A.: Proton translocation and the respiratory nitrate reductase of *Escherichia coli*. Biochem. J. **152**, 547–559 (1975)

Gutowski, S. J., Rosenberg, H.: Succinate uptake and related proton movements in *Escherichia coli* K12. Biochem. J. **152**, 647–654 (1975)

Hamilton, W. A.: Energy coupling in microbial transport. Adv. Microb. Physiol. **12**, 1–53 (1975)

Harder, W., Visser, K., Kuenen, J. G.: Laboratory fermenter with an improved magnetic drive. Lab. Practice **23**, 644–645 (1974)

Kaback, H. R.: Bacterial membranes. In: Methods in enzymology, Vol. 22 (W. B. Jakoby, ed.), pp. 99–120. New York: Academic Press 1971

Konings, W. N.: Localization of membrane proteins in membrane vesicles of *Bacillus subtilis*. Arch. Biochem. Biophys. **167**, 570–575 (1975)

Konings, W. N., Freese, E.: L-Serine transport in membrane vesicles of *Bacillus subtilis* energized by NADH or reduced phenazine methosulfate. FEBS Lett. **14**, 65–68 (1971)

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275 (1951)

Matin, A., Konings, W. N.: Transport of lactate and succinate by membrane vesicles of *Escherichia coli*, *Bacillus subtilis* and a *Pseudomonas* species. Eur. J. Biochem. **34**, 56–67 (1973)

Mitchell, P.: Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Bodmin, Cornwall: Glynn Research Ltd. 1966

Mitchell, P.: Vectorial chemistry and the molecular mechanism of chemiosmotic coupling: Power transmission by proticity. Biochem. Soc. Trans. **4**, 399–430 (1976)

Moyle, J., Mitchell, P.: Electric charge stoicheiometry of calcium translocation in rat liver mitochondria. FEBS Lett. **73**, 131–136 (1977)

Padan, E., Zilberstein, D., Rottenberg, H.: The proton electrochemical gradient in *Escherichia coli* cells. Eur. J. Biochem. **63**, 533–541 (1976)

Papa, S.: Proton translocation reactions in the respiratory chains. Biochim. Biophys. Acta **456**, 39–84 (1976)

Quayle, J. R.: Metabolism of C_1 compounds in autotrophic and heterotrophic microorganisms. Ann. Rev. Microbiol. **15**, 119–152 (1961)

Ramos, S., Kaback, H. R.: The electrochemical proton gradient in *Escherichia coli* membrane vesicles. Biochemistry **16**, 848–853 (1977)

Ramos, S., Schuldiner, S., Kaback, H. R.: The electrochemical gradient of protons and its relationship to active transport in *Escherichia coli* membrane vesicles. Proc. Nat. Acad. Sci. U.S.A. **73**, 1892–1896 (1976)

Rottenberg, H.: The driving force for proton(s) metabolites co-transport in bacterial cells. FEBS Lett. **66**, 159–163 (1976)

Stroobant, P., Kaback, H. R.: Ubiquinone-mediated coupling of NADH dehydrogenase to active transport in membrane vesicles from *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **72**, 3970–3974 (1975)

Received June 6, 1977